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FINAL REPORT

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THE UTILIZATION OF BLOOD PLASMA PROTEIN AS A HIGH-INTENSIFY FLAVOURING AGENT

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SASKATCHEWAN
FOOD PRODUCT *Innovation* PROGRAM
PRODUCT DEVELOPMENT AND RESEARCH FOR MEAT PROCESSORS

The Utilization of Blood Plasma Protein as a High-Intensity Flavouring Agent

Agriculture Development Fund
Project # 98000076

Final Report

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ABSTRACT

This project investigated a value-added application for defibrinated plasma from bovine blood by evaluating its performance as a substrate for reaction flavour development. The proteins of defibrinated bovine plasma (DBP) can be hydrolysed with food grade microbial proteases but only when proteins are heat denatured. Free amino acids and small peptides obtained from hydrolysis of plasma proteins participated in the Maillard reaction with reducing sugars when suitable conditions were provided. This sugar-amino reaction produced volatile compounds possessing aroma notes of cooked meat when free cysteine and thiamine were present in the medium. This study shows a direction for utilization of DBP; it has a potential for application in a number of food products.

EXECUTIVE SUMMARY

This project was proposed to find a value-added use for bovine blood plasma, an underutilized co-product of the animal slaughter industry. The collected bovine blood is processed to recover plasma and red blood cells. Selective removal of fibrin and thrombin proteins from the plasma (mainly from bovine blood) is a patented process and a commercial plant to process these plasma proteins has been established in western Canada. This protein separation process leaves behind defibrinated bovine plasma (DBP) containing 6 to 7% protein which has some utility in emulsion type meat products as a protein supplement. DBP is a very economical source of animal protein and has a potential to be used as a substrate to develop into flavour compounds.

The experiments for this project were carried out in three major phases. In phase one, suitable enzymes and reaction conditions for plasma protein hydrolysis were evaluated. In the second phase, the hydrolyzed plasma protein was characterized. Development of reaction flavours and characterization of their volatile compounds were performed as the third phase of the project. In order to meet the project objectives several experiments were carried out and different analytical techniques and instrumentation were employed for each phase.

Conversion of plasma proteins into a suitable substrate containing free amino acids and peptides for flavour-generating reactions was achieved via enzyme-assisted hydrolysis. Enzymatic hydrolysis was employed, as it is relatively easy to control and produces minimum amounts of unwanted side products compared to chemical-assisted hydrolysis. It was found that DBP should be thermally treated as most plasma proteins become susceptible to enzymatic hydrolysis when heat denatured. Two food grade microbial enzymes (Alcalase®

and Flavourzyme™) were studied and Flavourzyme resulted in a very high degree of hydrolysis (DH%) for DBP proteins. Use of Flavourzyme was advantageous, as this particular enzyme requires natural pH drift for optimum activity compared to static pH condition, which Alcalase needs. Therefore, a base addition was necessary to adjust pH at the start of hydrolysis; this was the only chemical added to the plasma besides the enzyme. Optimum reaction conditions that give extensive hydrolysis of plasma proteins with Flavourzyme were established. Overall this hydrolysis process utilized a minimum amount of chemicals, required common unit operations such as heating and mixing, and could easily be scaled up to prepare large volumes of hydrolysed DBP. Since the starting DBP has a very high content of water, vacuum evaporation can be used to increase the total solids content thus increasing the reactant concentration for further utilization. Before hydrolysis, DBP appeared as a light red coloured liquid and turned into a straw coloured liquid due to heat treatment and hydrolysis.

Since the amount of free amino groups available after hydrolysis depends on the extent of hydrolysis, a very high degree of hydrolysis was sought for this study. High values of DH% and free amino acid content are the indicators of degradation of proteins due to Flavourzyme activity. Results of chromatographic separation of hydrolyzed proteins showed that the constituent protein molecules were broken down and small molecules mostly free amino acids and peptides were formed on account of hydrolysis. Before hydrolysis, all proteins were of molecular weight greater than 26.8 kDa and enzymatic hydrolysis converted a considerable fraction of molecules to be less than 1.04 kDa.

Hydrolysis of DBP proteins resulted in a substrate suitable for sugar-amino reaction that could generate flavour volatiles similar to which happens during thermal processing of

meat. D-ribose and D-glucose, which are the predominant reducing sugars found in post rigor meat, were used for flavour generation reactions with the amino groups of hydrolysed DBP. When only these sugars (i.e., D-ribose or D-glucose) were added to hydrolyzed proteins and heated at 119°C to go through Maillard reaction, recognizable "meaty" aroma was not developed. Addition of free cysteine and thiamine was needed to create a suitable mixture of reactants for formation of volatiles possessing meaty aroma. The overall aroma perceived from these reaction mixtures was described as sulfurous, meaty, burnt meat, bouillon, cooked chicken like, cooked pork like, cooked liver like, caramel and fishy by a panel of nine semi-trained assessors. Results of gas chromatographic separation also confirmed that mostly sulphur containing compounds, alkyl substituted heterocycles and alkyl thiophenes constitute the volatile components of these reaction mixtures. These are the predominant components of boiled or roasted meat. The process of flavour generation employed here is similar to what commercial producers use for developing flavourants with hydrolyzed animal or plant proteins.

This study shows an avenue to utilize DBP by modifying its proteins. The flavourings that were developed have potential to be included in liquid type flavourants as they have high moisture content. Drying or concentration of the final product may be possible, but it would add extra cost to the final product. Development of flavour compounds is not the only possibility for utilizing hydrolysed proteins of DBP. As the produced DBP protein hydrolysate has minimum chemical additives, it may be suitable for several other applications that utilize constituent peptides and amino acids.

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1. INTRODUCTION

Blood comprises about 6% of the live weight of cattle and it is a highly proteinaceous by-product. Blood proteins are approximately 10% of the total protein content of the animal. Effective removal of blood during slaughter is required for improved colour and keeping qualities of the carcass. Approximately 50% of the animal's blood can be collected during bleeding in the slaughter operation and the remaining is retained in the capillary system through out the body. About 0.5 kg of pure protein can usually be salvaged per beef animal. Blood is collected hygienically and then anticoagulant, usually trisodium citrate (0.2% with or without water) is added. Processing of blood includes centrifugation to separate light plasma (52-70%) from heavy erythrocytes and then chilling to 2°C if not previously done to minimize bacterial growth. Plasma may be used to obtain different types of proteins with biochemical significance. These fractions or whole plasma may be frozen as flakes and distributed or stored at frozen stage (Wismer-Pedersen, 1979; Ockerman and Hansen, 2000).

The basic units of proteins are amino acids that are linked together via peptide bonds to form constituent peptides. Any chemical or physical activities that can break one or more peptide bonds are capable of hydrolysing the protein. Most of the food protein hydrolysis studies have focused on using the final product for enhanced properties such as, functional ingredients, dietetic foods and flavouring agents. Acid or base-catalysed chemical hydrolysis has been long practiced to obtain protein hydrolytic products as flavour precursors such as in hydrolyzed vegetable (HVP) and hydrolyzed animal (HAP) proteins (Nagodawithana, 1995). The so obtained hydrolyzed proteins are used as savory ingredients since they contain potent flavourants such as free amino acids and their sodium salts and peptides or used in process-

flavour generation via chemical reaction with reducing sugars. Hydrolysis of proteins using acid catalysis is very efficient but the difficulty of controlling hydrolysis of other organic materials in the substrates has been a problem. The major concern is the presence of monochloropropanal (3-chloro-1,2-propanediol, 3-MCPD) and dichloropropanal (1,3-dichloro-2- propanol, DCP) from lipid hydrolysis (Collier and others, 1991; Velisek and others, 1991) and sodium salts of glutamic acid (MSG) in the neutralized final product (Nagodawithana, 1995). Peptidyl hydrolases that have the ability to break peptide bonds are favoured in the food industry. Enzyme catalyzed proteolysis has widely been applied for plant, dairy and other animal proteins and is a very useful tool for improving the utility of a protein with a limited or single use. Use of proteolytic enzymes to hydrolyse proteins avoids unwanted side reactions thus preventing formation of 3-MCPD and DCP in the final product. Therefore, enzymes, which specifically catalyze only the hydrolysis of proteins, have been the choice for hydrolytic modification of proteins, which are intended for food use.

Flavorings that mimic "cooked meat" are used to enhance or impart meaty-flavour in convenience and processed foods and are accepted as part of our diet. These flavorings are also required to provide flavour in meat analogues. Widely used meat analogues may be partial meat substitutes to achieve lower retail prices or non-meat ingredients in vegetarian diets and diets with low levels of cholesterol and saturated fats (reduced/low fat products). Convenience foods such as instant noodles or pasta with dried sauces also utilize such flavorings including simulated cooked meat of a variety of species. Consumers expect these products to taste like meat or broth; therefore, flavourings that potentiate meat like aroma are widely employed to provide cooked-meat flavour.

Use of amino acid mixtures based on hydrolysis of animal or plant proteins to improve meaty-flavour of thermally processed foods is a common practice and also has a sound chemical rationale. The free amino acids, peptides and sugars are the key precursors in generating flavour of cooked meat regardless of the species-specific flavour notes. Most of the compounds associated with the cooked meat flavour are generated via Maillard reaction. This key reaction of flavour generation is initiated by free amino group of amino acids/peptides and reducing end of hexose or pentose sugars present in post-rigor meat that react at high temperatures (involved in cooking). Protein hydrolysates derived from low-cost animal or plant protein sources are the most economical form of free amino groups for development of simulated meat flavour compounds based on the Maillard reaction. In reaction flavour development based on the Maillard reaction, the flavour intensity generally increases when the amino groups are in the form of small peptides and amino acids.

In the characterization of odour active compounds of food matrices, gas chromatography (GC) is the most suitable tool to separate and identify consisting molecules. GC combined with a flame ionization detector (FID) can separate volatile mixture into individual components and these components can be identified based on the retention time of known chemical standards. GC-mass spectroscopy (GC-MS) may provide information on structural identification of the compounds that are separated by GC. Olfactometry combined with GC (GC-O) is a technique in which the odour of eluting compounds separated from GC is evaluated by human subjects. Each odour active compound may be assigned odour descriptors as perceived by the human nose. In descriptive evaluation of sensory attributes quantitative

descriptive analysis (QDA) provides precise and accurate description of individual sensory attributes of a composite food by employing trained assessors.

The main objective of this project was to investigate a value-added application for defibrinated bovine plasma (DBP) by means of the following:

(1) Hydrolysis of DBP proteins with microbial proteases to obtain a protein hydrolysate.

(2) Development of thermal reaction flavours with DBP hydrolysate and reducing sugars directed towards cook-meat flavour.

(3) Evaluation of developed reaction flavour mixtures by instrumental and sensorial methods.

The project was carried out in three phases as to achieve above-mentioned objectives. Details of the experiments and results of these three phases of the project are reported as individual chapters in the report.

2. PHASE 1- HYDROLYSIS OF BLOOD PLASMA PROTEINS

Literature Review

Blood comprises a considerable fraction of abattoir waste biomass in the animal slaughter industry. Plasma is the liquid part of blood once the red blood cells are separated (treated with anticoagulants and then centrifuged). Liquid plasma constitutes about 63% of weight of total blood, of which approximately 7% is protein. Different food applications of blood plasma have been investigated in the literature.

Proteins of blood plasma have been used to prepare meat analogues by texturization (Young and Lawrie, 1974) and also have been included in sausage and puddings in their dried form (Wismer-Pedersen, 1979; Cadironi and Ockerman, 1982a,b; Guzman and others, 1995). Complexation of plasma proteins with metaphospahates permits the isolation of these proteins and such protein isolates are suitable for different food applications (Etheridge and others, 1981). Bovine blood plasma proteins may be fractionated in order to obtain immunoglobins, fibrinogen and serum albumin to use as food or feed ingredients (Donnelly and Delaney, 1977; Lee and others, 1987). Blood plasma proteins possess good functional properties such as gelation and emulsification (Wismer-Pedersen, 1979; Howell and Lawrie, 1984a,b; 1987; O'Riordan and others 1979; Guzman and others, 1995; Cofrades and others, 2000). Ishiguro and Ichikawa (1975, 1977) have patented a process to utilize dried blood plasma solids to generate beef like flavour by reacting the solids with ribose or ribose-5-phosphate at high temperature. About 150 different proteins with physiological significance have been identified from plasma (Anderson and Lunden, 1979). Several of these proteins in plasma have exhibited cross-linking ability of major muscle proteins (especially myosin) and protease

inhibitory activity thereby enhancing resistance to endogenous protease degradation (Saito and others 1992; Seymore and others, 1997). Therefore, blood plasma has been studied to improve gel strength of surimi products from certain aquatic fish species (Whitmore and Torley, 1988; Weerasinghe and others 1996; Kang and Lanier 1999). The process patented by Harimex B.V. of the Netherlands, selectively precipitates (use cryo-precipitation) fibrinogen and thrombin from animal blood plasma. This product is marketed as Fibrimex® and has been well utilized as a natural binder for whole muscle processing. Separation of these two proteins only, leaves behind plasma rich in albumin and globulin which is referred as defibrinated plasma.

The objective of this phase of the study was to utilize proteins of defibrinated bovine blood plasma as the substrate for exogenous enzymatic hydrolysis in order to obtain a final product containing reactants (e.g., free amino acids and small peptides) for process flavour generation.

Experimental

Materials

Defibrinated bovine blood plasma (DBP) was obtained from Harimex B.V. (formerly FNA Foods, Strathcorna, Alberta) as frozen flakes (stored at -30°C) and used through out the study. All chemicals used were of ACS grade. The microbial enzymes Alcalase 2.4L® and Flavourzyme L™ were obtained from Novo Nordisk Company (A/S Denmark). Alcalase is a microbial protease from *Bacillus licheniformis* with endopeptidase activity and had a declared activity of 2.4 Anson units (AU) per gram. Flavourzyme is a protease complex obtained from

fungi *Aspergillus oryzae* that contains both endo- and exoprotease activities and had a declared activity of 1000 leucine amino peptidase units (LAPU) per gram.

Methods

Preliminary experiments showed that bovine plasma proteins in the native state were not susceptible to hydrolysis by Alcalase or Flavourzyme. Therefore, heat denaturation of the proteins was carried out at 95°C for 15 min in a water bath.

Chemical Composition

The contents of moisture and total nitrogen of the DBP samples were determined according to AOAC (1990) methods. The dry matter content was calculated as 100 - percentage moisture content. Total lipid content was determined according to Bligh and Dyer (1959).

Measurement of Degree of Hydrolysis

The degree of hydrolysis defined as the percentage of peptide bonds cleaved was calculated based on the determination of free amino groups by the *o*-pthaladehyde (OPA) method (Church and others. 1985: Van Beresteijn and others. 1994). The amount of free amino groups was calculated as serine-NH_2 groups. Acid hydrolysis of DBP in 6 M HCl for 24 h at 110°C was carried out for determination of total free amino groups of plasma proteins. The degree of hydrolysis (DH%) was calculated as the ratio of released free amino groups due to enzymatic hydrolysis to total free amino groups released due to complete acid hydrolysis per unit of protein and expressed as a percentage value.

$$\text{Degree of Hydrolysis (DH\%)} = \frac{\text{Free amino groups released due to enzymatic hydrolysis}}{\text{Total free amino groups from acid hydrolysis}} \times 100$$

Enzymatic hydrolysis

The actual set up of hydrolysis is shown in Figure 2.1. The flow chart provided in Figure 2.2 explains the basic steps involved in the hydrolysis process of DBP. Control experiments were carried out without enzymes. Both Alcalase and Flavourzyme were studied during the first stage of experiments. Only Flavourzyme was used for the optimization study of hydrolytic conditions. In the enzyme hydrolyzed samples the DH values were determined as mentioned in the above section.

Optimization of hydrolytic conditions

A two factor central composite rotatable design was employed to study the response, DH of blood plasma protein by Flavourzyme as changed with the concentration of enzyme (LAPU/g protein, X_1) and time of hydrolysis (min, X_2). Experimental data were analysed for response surface regression according to Wanasundara and Shahidi (1996) for a quadratic polynomial model using SAS software (SAS Institute Inc., 1990).

Results and Discussion

Enzymatic Hydrolysis

The general composition of unhydrolysed DBP is given in Table 1.1. The mean protein content based on Kjeldhal nitrogen was 6.13% (6.25xN%) and it was the predominant component of blood plasma dry matter. Figures 2.3A and B show the hydrolysis pattern of plasma proteins by Alcalase and Flavourzyme as the time of hydrolysis and enzyme

concentration changed. In the native state DBP showed a resistance to both these enzymes and gave very low values of degree of hydrolysis (DH%). Thermal treatment of DBP improved the DH values for both enzymes most probably due to heat-induced denaturation of proteins. The liquid DBP formed a translucent light yellow coloured gel due to thermal treatment and upon hydrolysis the gel again turned into a free flowing liquid.

Table 1.1. General characteristics of bovine blood plasma.

Characteristic	Value
pH	8.00 ± 0.20
Density	1.030 ± 0.010 g/ml
Dry matter content	7.98 ± 0.020 %
Total nitrogen content	0.98 ± 0.10%
Non-protein nitrogen content	0.05%
Total lipid content	0.5%

When the two enzymes are compared for their ability to hydrolyse DBP proteins (Figure 2.3), Alcalase gave a maximum of 24.8±1.5% DH at the highest enzyme concentration (0.5 AU/g protein) and prolonged hydrolysis (24 h). However, Flavourzyme was more efficient in hydrolysing DBP and gave DH of 48.3 ± 2.3% at 24h when 100 LAPU/g protein was used. It has been well documented that Alcalase, which is a bacterial endoprotease, is limited by its specificity and depending on the substrate the highest DH that can be reached is 20-25% (Alder Nissen, 1986, Pommer, 1995). However, fungal protease complex

Flavourzyme has a more broad specificity, thus when combined with its exopeptidase activity high DH values can be achieved, perhaps even 50% giving mostly dipeptides in the hydrolysate (Pommer, 1995). Therefore, in the production of hydrolyzed-plant or-animal protein Flavourzyme has the advantage over Alcalase as non-bitter peptides are formed in the hydrolysates (Anon, 1996). During the course of hydrolysis a natural pH drift (from 7.0 to 5.0) rather than maintaining a stable pH (pH-stat) is favourable for optimum activity of Flavourzyme. This is an advantage for the present study as no basic reagents (NaOH) was used to stabilize pH, thus avoiding formation of sodium salts of free amino acids. Also it minimizes the amount of chemicals in the final hydrolysis product. Termination of enzymatic action was achieved by thermal treatment (95°C for 3 min) to avoid any use of chemicals. In a single batch of hydrolysis a maximum of 600 ml of hydrolyzed DBP was prepared using the laboratory set up shown in Figure 2.1. The primary objective of this phase of the study was to hydrolyse major proteins of plasma in order to obtain peptides and free amino acids, which are the primary precursors of generating process-flavour upon reaction with reducing sugars. Therefore, high values of DH were preferred and the hydrolysis study was continued with Flavourzyme. The hydrolyzed DBP mixture at the final stages was a liquid and separation or filtration was not carried out, thus it was assumed that in hydrolyzed samples the total contents of dry matter and nitrogen remained same as in the original unhydrolysed sample.

Optimization Study

Figure 2.4 represents the response surface plot for DH% as affected by duration of hydrolysis and concentration of Flavourzyme. It shows that a maximum value of hydrolysis

could be achieved at 15.5h of hydrolysis using 110 LAPU/g protein. Prediction equation using the coefficients obtained from data analysis is as follows:

$$DH\% = -18.663 + 0.551\,[\text{LAPU/g protein}] + 0.066\,[\text{min}] - 3.06 \times 10^{-3}[\text{LAPU/g protein}]^2 - 4.36 \times 10^{-5}[\text{min}]^2 + 1.30 \times 10^{-4}[\text{LAPU/g protein}]\,[\text{min}]$$

This equation was useful in estimating the DH% value when a particular enzyme concentration and hydrolysis time was used and was employed in the rest of the study.

Summary

Heat denatured DBP proteins can be hydrolyzed with Alcalase or Flavourzyme. Up to 43% DH was obtained from Flavourzyme under the optimum conditions. Hydrolysis process of this enzyme utilizes minimum amount of chemicals and the process could be easily scaled up to hydrolyse large volumes of substrate. The hydrolyzed plasma protein has a good potential to participate in flavour generation reactions, as it is rich in small peptides.

3. PHASE 2 - CHARACTERIZATION OF HYDROLYZED PLASMA PROTEINS

Literature Review:

Hydrolyzed proteins from economical sources, especially food industry by-products have many valuable uses. Such uses include, nutritional supplements, functional enhancers for composite foods, flavourants, or substrates for further reactions (e.g., process flavour generation). The degree of protein hydrolysis, peptide size (molecular size) and peptide composition dictate the overall functionality of the hydrolyzed protein. There is considerable evidence that free amino acids and peptides formed during protein hydrolysis serve as volatiles that provide flavour notes to thermally processed foods.

Degree of hydrolysis (DH%) determines to what extent the protein is broken down. It is estimated based on either determination of the amount of nitrogen released due to hydrolysis and which became soluble in the presence of a precipitating agent, determination of free $-NH_2$ groups or titration of the released protons (Silvestre, 1997). In a hydrolyzed protein intented for food applications, the knowledge of molecular weight distribution is important since the peptide type/group and size dictate the potential taste (e.g., bitter, sweet, umami) of the hydrolysate, volatile compounds generated during Maillard reaction, allergenicity, opoid or any other biological activities. Peptides play a significant role in aroma generation of cooked meat, hydrolyzed vegetable proteins and roasted cocoa and coffee. Different peptides with varying sequences could serve as precursors for the production of a variety of food aromas. It has also been noted that size, sequence and chemical conformation of peptides are significant contributing factors when attempting to produce specific aroma compounds via Maillard browning (Izzo et al., 1993).

In order to know the composition of peptides in the hydrolysate, separation and identification of the constituent peptides and amino acids has to be carried out. However, it is very difficult to know the composition of the protein hydrolysate because of the large number of possible constituents. This is due to the multiple degree of polymerization of peptides characterized by the 20 naturally occurring amino acids. The objective of this part of the study was to profile the hydrolyzed DBP based on the size of their molecular constituents by employing different chromatographic techniques.

Experimental:

Materials

Hydrolyzed DBP protein was obtained as described under Methods, Phase 1. To obtain DBP with different DH%, hydrolysis was performed using the prediction equation developed in Phase1 to estimate enzyme concentration and hydrolysis time. Free amino groups before and after hydrolysis were determined using the *o*-pthalaldehyde method and used to calculate and confirm the DH% value. Hydrolyzed DBP so obtained was lyophilized and used for all the experiments of this phase.

Methods

Analysis of Amino Acids

For determination of total and free amino acids, DBP samples were prepared by performic acid oxidation according to the method by Llames and Fontaine (1994). For determination of free amino acids, samples were prepared according to Shahidi and others

(1993). The individual amino acids in those samples were separated and quantified using a Beckman System Gold HPLC system (Beckman Instruments Inc. Fullerton, CA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Proteins

Tris-Glycine/SDS-PAGE was performed according to the method of Laemmli (1970). Proteins were separated with 10% resolving and 4% stacking polyacrylamide gels (100mm x 70mm x 0.75mm) on a Mini-Protean III system (Bio-Rad laboratories Canada Ltd. ON). The gels were run at 150 V constant voltage at 15°C and stained with Coomassie blue R-250 (in 50% v/v methanol and 10% v/v acetic acid), destained with 40% (v/v) methanol and 10% (v/v) acetic acid and stored in 10% v/v acetic acid and 5% (v/v) glycerol. The wide range molecular weight markers were also ran in the same gels.

Peptides

Tris-Tricine/SDS-PAGE was performed for separation of hydrolyzed BPP samples and carried out according to the method of Schägger and von Jagow (1979). Polyacrylamide gels of 16.5%T, 3%C were used as separating gels and stacking gel was 4%T, 3%C. The composition of running buffers were, 0.1 M Tris, 0.1 M Tricine and 0.1% SDS at pH 8.0 for cathode buffer and 0.2 M Tris-HCl at pH 8.9 for anode buffer. The gels were run at 110V and then fixed in 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min before staining (0.025% Coomassie blue G-250 in 10% v/v acetic acid for 1 h). Destaining was carried out in 10% (v/v) acetic acid solution until desired background colour was achieved. Polypeptide molecular weight standards (Bio-Rad Lab Canada, Missisauga, ON) were also applied with the samples.

High-pressure liquid chromatography

Reversed-phase chromatography

Samples of 2mg/ml was prepared in the mobile phase and filtered through a 0.45 µ (Amicon) membrane. A 20 µl sample was injected into a HPLC system equipped with a Shimadzu LC-10AD pump, SCTL 10A system controller, SPD-M 10A photodiode array UV-VIS detector, Millipore automatic sample injector, CTO-10AS column oven and CLASS-VP 5.x software for data handling. A C18-Hypersil Bakerbond BDC, 4.6 x 250mm, 5µ column was used for separation of peptides. Flow rate of 1ml/min, injection volume 20 µl and gradient elution of 0.1% TFA in water (solvent A), 0.1% TFA in 40%, v/v acetonitrile (solvent B), 0 to 5 min solvent A, 5 to 35 min solvent B from 0% to 40%, 35 min to 45 min 40% solvent B and 45 to 55 min solvent A was employed. Detection of peptides was at 220nm.

Size exclusion chromatography

The HPLC equipment consisted of a Waters 715 Ultra WISP sample processor, Waters 600 system controller, Waters 996 photodiode array UV-VIS detector, Millipore automatic sample injector and Millenium software for data handling. A TSK-G2500-PW column (7.5 x 300mm, 10 µ, TOSOHASS, Montgomeryville, PA) was used and elution solvent was 40% (v/v) acetonitrile containing 0.1% (v/v) triflouro acetic acid. Flow rate was 0.2ml/min and the injection volume was 20 µl. Detection of peptides was at 220 nm.

Results and Discussion

Amino Acid Composition

Table 2.1 provides the composition of total amino acids of unhydrolysed DBP and free amino acids of Flavourzyme-hydrolyzed DBP. The amino acid composition of bovine plasma proteins agrees with the previous reports (Lee and Others, 1987; Duarte and others, 1999), and is most similar to the amino acid composition of serum albumin. Glutamic acid, lysine, leucine, aspartic acid and arginine are the dominant amino acids and comprise more than 50% of the total amino acids. Since the total nitrogen content of DBP samples before and after hydrolysis remained unchanged its free amino acid composition was determined to evaluate the amount and type of amino acids released due to hydrolysis. Free amino acids may be the most potent reactants for process flavour development other than peptides. In the hydrolyzed samples, the most abundant amino acids in the free form followed the order of leucine, lysine, arginine, serine and phenylalanine, which is different than the order of abundance in the intact molecular protein. This order does not change with the degree of hydrolysis. It shows that more hydrophobic amino acids were released due to Flavourzyme hydrolysis than hydrophilic ones. Release of cysteine and methionine was minimum indicating that the content of free S-amino acids in the hydrolyzed samples was very low. There was a strong positive relationship (r^2=0.861) with the total amount of free amino acids and DH% values of the samples. The distribution of free amino acids can influence the flavour of hydrolyzed protein as well as the availability of amino groups that directly participate in the amino-sugar reaction to form volatile flavour compounds.

Table 2.1. Amino acid composition of unhydrolysed and Flavourzyme- hydrolyzed DBP.

Amino acid	Total amino acids[a] unhydrolysed DBP	Free amino acids[b]			
		0%[c]	12%	29%	48%
Ala	4.55	0.04	0.28	0.72	1.50
Arg	7.40	0.02	1.09	2.92	4.59
Asp+Asn	9.62	0.00	0.10	0.37	0.91
Cys	3.80	0.00	0.05	0.09	0.12
Glu+Gln	14.7	0.03	0.18	0.53	1.53
Gly	3.28	0.02	0.13	0.31	0.73
His	4.20	0.03	0.64	1.64	2.84
Ile	2.77	0.04	0.39	0.87	1.62
Leu	9.78	0.08	1.70	4.53	7.29
Lys	11.5	0.04	1.06	2.65	5.13
Met	0.98	0.00	0.01	0.03	0.05
Phe	5.20	0.05	1.15	2.54	4.24
Pro	2.78	0.00	0.00	0.00	0.51
Ser	6.70	0.03	0.86	2.19	4.30
Thr	6.34	0.04	0.60	1.43	2.94
Val	6.28	0.05	0.48	1.51	3.18
Total	99.81	0.47	8.35	22.32	41.45

[a]As mg/100mg of protein

[b]As mg/100 mg of protein

[c]Degree of hydrolysis

All values are average of duplicate samples

SDS-PAGE Study

Tris-glycine SDS-PAGE patterns of unhydrolysed, heat denatured and hydrolyzed BPP are presented in Figure 3.1. In the unhydrolysed sample, 6 major protein bands were identified, 2 bands between, 200 to 97.4 kDa, 3 bands between 66.7 to 45 kDa and one band between 21.5-14.4 kDa. There have been several reports on fractionation of blood plasma protein from animal processing industry and the major components are albumin (42%), globulins (α, β and γ, 53%) and fibrinogen (5%) (Howell and Lawrie, 1984; Lee and others, 1987).

Hydrolysis with both enzymes produced no or very few intact proteins as exhibited in Figure 3.1 (lanes 3-5 and 6-8 for Alcalase and Flavourzyme, respectively), which no bands appeared in Tris-glycine SDS- PAGE. However, a different separation was achieved when Tris-tricine-SDS-PAGE was employed. Figure 3.2 provides electrophoresis pattern (Tris-tricine SDS-PAGE) for Flavourzyme hydrolyzed DBP at different DH% values. It shows that for both the enzymes even at the highest DH% achieved in this study a single band equivalent to serum albumin was still remained. At very high DH% the intensity of the band was less. Serum albumin is the major protein in blood; therefore, certain amount of unhydrolysed molecules may have remained in the sample. For hydrolyzed DBP overlapping of numerous peptide products with the neighboring bands may have caused diffusion and no clear bands are visible. As DH% values increased above 15% (for both enzymes) most of the molecules of the sample were below 16.9 kDa. At the highest values of DH (24.0% for Alcalase 48.0% for Flavourzyme) only two major bands are present, one at 66.7 kDa and the other between 6.5 and 1.4 kDa, which migrated out of the gel. The lower intensity of these two bands than other

samples indicate that most of the molecules at high DH are lower than 1.4 kDa. Tris-tricine SDS-PAGE poorly resolves molecules lower than 1 kDa (Schägger and von Jagow, 1979). The tricine-SDA-PAGE gels show that Flavourzyme is able to break plasma proteins even at very low DH% values.

The fuzzy band detected on the 43% DH sample was compared with the peptide markers and it was estimated to have molecular weight close to1000 Da. Therefore, most of the molecules at this stage of hydrolysis could be of less than 1000 Da as the gel did not retain them.

HPLC separation

Separation of the DBP hydrolysate using RP-18 and TSK G2500PW are as in Figures 3.3.1 and 3.3.2, respectively. The RP-18 column separates molecules based on their hydrophobicity/hydrophilicity. The compounds eluted earlier in the chromatogram with this column are more hydrolphilic than the compounds later eluted. Unhydrolysed DBP (3.3.1A) has few compounds resolved during the separation and by comparing the peak heights; they were in small quantities as well. As DH% increased more hydrophilic components were formed and also few hydrophilic ones appeared (Figure 3.3.1B-D). TSK G2500PW is a size exclusion column and separates molecules mainly based on the molecular size. The unhydrolysed plasma protein molecules elute as one big peak from this column (Figure 3.3.2A). Generally larger molecules elute earlier in the chromatogram while small molecules are eluted later. As the hydrolysis progresses large protein molecules were broken down to small ones and separated on this column (Figure 3.3.2B-D). When peptides of known molecular weight were separated on the same column, molecules of 1040 and 369 Da eluted at

40 and 49 min, respectively. The dominant peak of 43% DH sample eluted at 52 min. For the same sample there are several components resolved between the retention time of 40 and 50 min (Figure 3.3.2D). Therefore, most of the molecules at this stage of hydrolysis may have molecular weight between 300 and 1000 Da.

Summary

According to chromatographic separation, the hydrolytic process has broken down most of the large protein molecules of DBP. Despite the relative high enzyme concentration and extended hydrolysis time the main products of DBP hydrolyzed with Flavourzyme were peptides rather than free amino acids. Therefore, it is expected that this hydrolytically modified DBP has a suitable molecular profile for tailor-made process-flavour generation.

4. PHASE 3 - DEVELOPMENT AND CHARACTERIZATION OF REACTION FLAVOUR

Literature Review

Improving flavour of food using hydrolyzed proteins has a long history. The best example is use of soy sauce obtained from hydrolytic action on denatured soy proteins (steamed soybeans), which is very popular in the orient. Hydrolysis of proteins produces free amino acids and peptides that have peculiar taste properties rather than aroma. However, in generation of cooked meat flavour hydrolytic components of proteins play a different role. The early works of meat flavour showed that water soluble, relatively low molecular weight components are the precursors of meat flavour. These work indicated that amino acids, peptides and very low molecular weight carbohydrates are among these precursors. The cold-water extract of beef contained a compounds less than 200 Da including various free amino acids (cysteine, alanine, glutamic acid and tryptophan), glucose, glucosamin and ribose. It was also found that ribose and cysteine disappeared completely on thermal treatment which indicated that they were involved in reaction flavours (Macleod, 1994).

Peptides have long been recognized as important compounds in process-flavour development. Besides the participation in flavour generation reactions, free amino acids and peptides have distinct flavour profiles. Peptides contribute to volatile aroma compound formation under thermal reaction conditions and certain amino acids in the bound form of peptides undergo Strecker degradation to form Strecker aldehydes in Maillard reaction (Rizzi, 1989; Zang and others 1992). Model system studies have proved that the peptides are more reactive than free amino acids in participation in Maillard reaction when glucose was used as the sugar (de Kok and Rosing, 1994). The reactivity of peptides decreases with the increased

chain length for homopolymers and presence of side-chain carboxylic residue (e.g., glutamic acid) in a dipeptide enhances the reactivity. Compared to acid-hydrolyzed vegetable or animal protein, enzyme catalysed protein hydrolysates contain a larger portion of peptides than free amino acids owing to the high selectivity and specificity of proteases (de Kok and Rosing, 1994).

In meat, low-molecular weight peptides are readily formed through endogenous proteolysis of larger meat proteins (Spanier and others, 1988) that participate in peptide-sugar reaction during thermal processing and are potential flavour precursors. There are about 1000 aroma compounds identified from cooked-beef alone. However, no single compound uniquely responsible for cooked beef aroma has been identified, although some have been described as "meat like". Most of the volatiles generated during thermal processing contribute to overall aroma of cooked meat. The character impact compounds are the most important contributors to the specific aroma note and generally have low threshold values.

In order to characterize or identify volatile compounds of foods a concentrated form of volatile isolate is needed. Classical steam distillation based simultaneous extraction may allow volatiles to escape from the food matrix and dissolve into non-polar organic solvents. Such non-polar solvents or their mixtures can be directly injected to a gas chromatograph for separation into individual components. Gas chromatography provides the best performance of all separating procedures and when dealing with the solutes in the vapour phase it is ideally suited to study the flavour volatiles. The chemical identity of the separated compounds can be deduced from the retention indices and mass spectroscopic analysis when combined with a mass detector.

Sensory profiling is very helpful in describing a particular aroma of food. Since the overall aroma is a blend of several volatiles, depending on the concentration and odour activity of each compound the human nose is able to recognize individual traits of aroma as a stimulant and also to quantify based on a scale. Quantitative descriptive analysis provides a mean of quantifying the perception thus allowing more scientific approach to evaluate the sensory quality of a product.

Experimental

Materials

Hydrolyzed DBP at 43% DH was used for flavour generation. All the chemicals used were of ACS grade. Commercial samples of roasted beef flavour were obtained from FIS Flavours, ON.

Methods

Flavour generation

Hydrolyzed DBP was concentrated under vacuum to increase total solid content. Concentration was carried out using a laboratory scale rotary evaporator (Buchi Rotavap, Switzerland). Total solid content of samples were determined gravimetrically and increased from 9 to 30%. Reaction mixtures were prepared mainly to contain DBP hydrolysate, D-glucose or D-ribose, cystine and thiamine in the ratio of 1.00 : 0.05 : 0.01 : 0.01 based on the weight of total solids of the hydrolysate. Glycine and inosine monophosphate was also used at

0.005 ratio. All the reactants were mixed very well and pH of the mixture was adjusted to 5.3 with 1% (v/v) lactic acid. Reaction flavour development was carried out at 119°C (as dry heat) until perceived aroma was developed. The duration of heating was different depending on the sugar used.

Extraction of volatiles

Reaction mixture (50 ml) and internal standard (3-heptanone, 10 ppm) was extracted for 120 min with pentane (5 ml) in a simultaneous distillation unit that is a Likens and Nickerson apparatus (Fisher Scientific, Napean, ON). Both the flasks containing sample and pentane (Kuderna-Danish concentrator tube) were maintained at boiling temperatures. After extraction the pentane layer was recovered and concentrated with a Snyder column down to 1 ml. The concentrated sample was used for GC analysis.

Gas chromatographic analysis of aroma volatile extracts

A GC-FID was used to identify compounds based on retention index and GC-MS to confirm the identity. One micro liter of each extract was injected into a fused silica capillary column (DB5 60 m length, 0.25mm I.D. 0.25 mfilm thickness, Supelco Inc, Bellefonte, PA). The linear velocity of helium carrier gas was 28.7 cm/sec. Oven temperature was programmed from 35 to 100 at 5°C/min with initial holding time of 5 min. Temperature was then further increased at 10°C/min to 250°C and held for 5 min. Duplicate analysis was performed for each extract. The system consisted of an HP-6890 GC/HP FID (Agillent Technologies, ON). Both the column inlet and detector were maintained at 260°C. Similar analytical conditions were used for GC-MS. A Hewlett Packard HP 6890 (Agillent technologies, Ontario, Canada) equipped with FID and Olfactory detector was used for retention index determination. A GC

(HP 5890) equipped with a mass detector, and operating at 70 eV, electron impact (EI) mode was employed for tentative identification of compounds.

Quantitative Descriptive Analysis of flavour reaction mixtures

Aroma profiling was carried out at the sensory evaluation lab, University of Saskatchewan. The panel was composed of nine assessors (semi-trained) with knowledge of cooked meat associated aroma. The descriptors for sensory profiling was initially discussed with the moderator. From the initial descriptors a set of reference standards was developed. The panel participated in total of nine training sessions including discussions within a ten-week period. For the final evaluation the assessors were presented 3 to 4 samples (3 digit coded) with the evaluation sheets containing descriptors and point scale. Samples were presented in 15 ml clear, screw capped vials and maintained at 50°C in a water bath (Figure 4.2). Panelists were asked to evaluate aroma and describe quantitatively for the descriptors (a sample sheet is provided in the Appendix). A set of reference standards (see Appendix) was also provided. A vial containing fruit flavored crystals was also provided as nasal refreshment between sample evaluation.

Results and Discussion

When Flavourzyme hydrolyzed-DBP proteins were reacted with reducing sugars (D-glucose or D-ribose), without any other additives, no significant aroma notes related to cooked meat developed. Only a strong aroma of drying plasma/blood with a slight caramel note was produced from these sugar-hydrolysate mixtures. The amino acid analysis indicated that proteins of DBP are low in S-amino acids and very low quantities are available in unbound form even after hydrolysis. Since S-containing amino acids are essential in developing cooked meat aroma L-cysteine was included to the reaction mixture. Another reaction mixture was developed with cysteine and thiamine, as naturally present thiamine (vitamin B2) in muscle tissues is essential in producing "meaty" aroma notes upon thermal degradation. When cysteine, thiamine and D-ribose are present in the reaction mixture aroma notes very close to cooked meat were developed, especially a meaty with strong sulfurous note. D-glucose containing reaction mixtures needed longer time to develop aroma notes at the temperature (119°C) that was most suitable for D-ribose. A separate reaction mixture that contained glutamic acid, glycine, histidine and inosine monophosphate and taurine in addition to cysteine, D-ribose and thiamine was also examined and was able to produce very intense "sulfury" and "meaty" notes. The over all aroma developed from this particular mixture has a strong meaty and sulfury aroma directed to odour notes of boiled or roasted beef. At the same time the individual volatile profiles of aroma extracts were monitored using GC-FID. About 30 compounds were separated from ribose containing reaction mixture and some of them are as in Table 3.1. All the compounds with retention time less than 4.5 min on the GC were removed, as they were confounded with the solvent. Only 24 compounds were tentatively identified

(Table 3.1) with GC-MS. Presence of five compounds was confirmed by retention time of the available authentic chemical standards. Presence of several sulphur-containing compounds and alkyl substituted heterocycles and thiophenes predominated in the reaction mixture indicating that the sugar-amino reaction had occurred and indeed the resulting compounds are responsible for sulfury aroma. The reaction mixture containing D-glucose also resulted similar compounds, but several other compounds were also detected. In contrast to the hydrolysate-sugar reaction mixtures the commercial sample of roast beef flavour contained more alkyl substituted pyrazines and different sulphur containing compounds.

Descriptive Analysis

A set of 9 descriptors was developed by the assessors to describe (consensus profiling) the aroma of these DBP hydrolysate containing reaction mixtures. The results of their evaluation are summarized as a radar chart (Figure 4.3) using the mean value for each descriptor. Sulfurous and meaty were the dominant traits of the aroma and burnt meat and bullion notes followed them. The panel also described that both the reaction mixtures have cooked chicken, pork or liver notes and all these scored very similarly. Caramel and fishy notes were detected by most of the panelists but had very low scores. When the commercial roast beef flavour sample was compared with reaction mixtures, burnt meat and bouillon was the predominant notes. All other descriptors scored values close to none. This sample had a distinct difference in the composition of volatiles as described in the above section. The pyrazines may be mostly responsible for the roasted, burnt notes.

Table 3.1 Constituents of volatile extracts of DBBP hydrolysate reaction mixture with D-ribose.

Chemical compound	Kovac Index[a]	Area relative to IS[b]	Identified by MS[c]
		D-Ribose	
3-Methyl butanal	802.9	102	+++
2,3-pentanedione	809.6	23.0	+++
2-Methyl thiophene	823.4	4.56	++
2-Methyl furan	834.7	32.7	+++
1-Pentanol	844.4	10.1	+
2,2,4,4,-tetra methyl pentane	866.2	19.5	++
Dihydro-2-methyl-3(2H) furanone	897.3	9.53	+++
2-Methyl-(1H) pyrrole	902.0	11.4	+
2-Methyl butanoic acid	908.8	7.25	++
5-Methyl-2(5H) thiophene	942.3	103	+
2-Methyl-3-pentanethiol	1045	54.8	+
2-Methyl amino-2-thioxoacetamide	1125	15.8	++
2-Furanmethanethiol	1207	21.7	++
2-Ethyl-1H-pyrrole	1249	5.60	+++
2-Acetyl furanone	1576	6.64	+
2-Butyl furan	1613	5.47	++
2-Pentylthio thiophenone	1678	7.73	+
2-Methyl tetrahydro thiophene-3-one	1687	5.8	+
Benzeneacetaldehyde	1715	20.83	+++
2-Thienyl methanol	1798	5.38	+
p-Cresol	1818	6.13	+
3-Methyl-2-thiophene carboxaldehyde	1953	12.4	++
2,4-Dimethyl-5-propylthiazole	1999	6.66	++
Isopropyl-4-ethyl-5-methyl thiazole	2118	12.87	+

[a] Determined with a retention index mix (C8-C32 hydrocarbons) using GC-FID

[b] Area relative to internal standard (IS) 3-heptanone, 10 ppm

[c] The quality of matching of mass spectroscopy (MS) results with MS database; 8 = +++, 7 = ++ and 6 = +.

Summary

Hydrolyzed DBBP can generate volatiles with cooked-meat related aroma when reacted with D-ribose or D-glucose, cysteine and thiamine. Removal of excess water in the plasma is preferable to direct reactions more towards favorable aroma. Both ribose and glucose developed predominantly sulfurous and meaty aromas notes reaction mixtures.

5. FUTURE RESEARCH DIRECTIONS

This study showed that defibrinated bovine plasma proteins could be hydrolysed and converted to voaltiles with flavour activity. More work could be carried out on flavour development. These studies could be oriented to:

- Studying different combinations of hydrolysed plasma and additives that were used in the experiments described in the present study. The variables could be degree of hydrolysis, concentration factor (total solid content), ratios of sugar with L-cysteine and thiamine. Since the methods to isolate and to characterize developed volatiles are established in this study, further experiments of this nature are very straightforward.
- In order to introduce species-specific flavour characters to the developing flavourant inclusion of animal fat (e.g., beef tallow, lard or chicken fat) to the reaction mixtures could also be studied. At the same time a method to stabilize such flavour composites should also be taken into consideration as lipids may induce off-flavour development with storage.
- Present study shows that plasma proteins can be used as a substrate to obtain peptides. Such peptides may have particular amino acid sequences that possess biological activities. This would be another promising area to study for further value-added utilization of DBP.
- Study of plasma proteins from other livestock species (e.g., avian, porcine, etc.) for such uses.

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7. PROJECT RELATED COMMUNICATIONS

Following communications were produced and are being prepared as scientific communications from this project.

Wanasundara, P.K.J.P.D., Pegg, R.B. and Shand, P.J. 2000. Preparation and molecular characteristics of hydrolysed bovine blood plasma proteins. 2000 IFT Annual Meeting and Food Expo of Institute of Food Technologists. June 10-14. Dallas, TX, USA.

Manuscripts in preparation:

Enzymatic hydrolysis of bovine blood plasma proteins
Proposed Journal: *Journal of Food Science*

Molecular characteristics of hydrolysed bovine plasma proteins
Proposed Journal: *Journal of Food Science*

Flavour compound generation from hydrolysed bovine plasma proteins:
Identification of aroma volatiles and their sensory characteristics
Proposed Journal: *Journal of Agricultural and Food Chemistry*

8. PERSONNEL FUNDED BY THIS PROJECT

M. Lafond	Technical assistance-casual	casual hours
J. Wanasundara	Food Scientist	15 person months

9. EQUIPMENT PURCHASES

No equipment valued at over $500 was purchased with funds of this project.

10. STATEMENT OF EXPENDITURES

The statement of expenditures as prepared by the University of Saskatchewan is presented in the Appendix.

11. ACKNOWLEDGEMENTS

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FIGURES

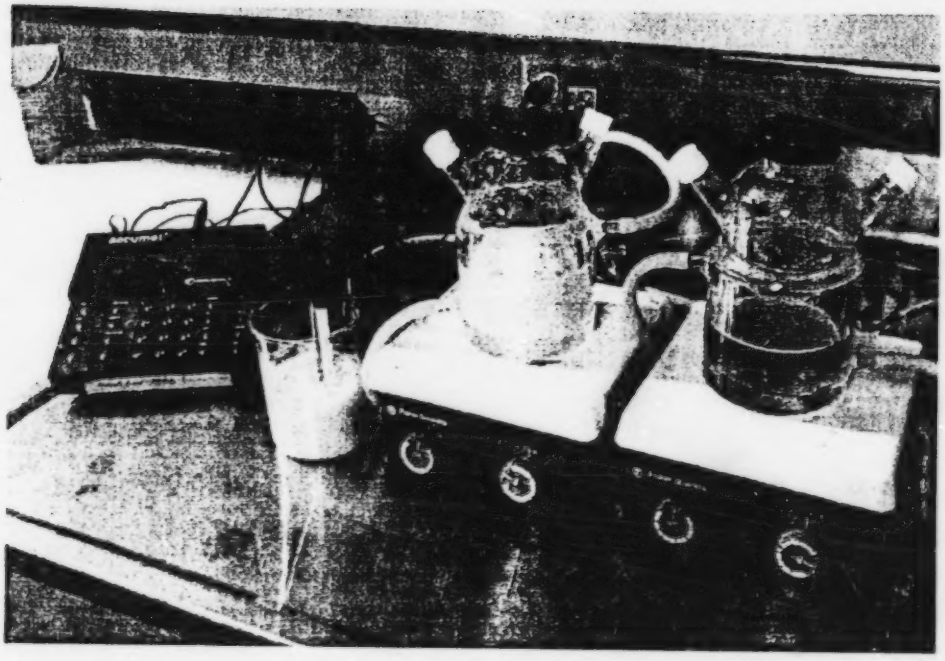

Figure 2.1 Experimental set up for defibrinated bovine plasma (DBP) hydrolysis.

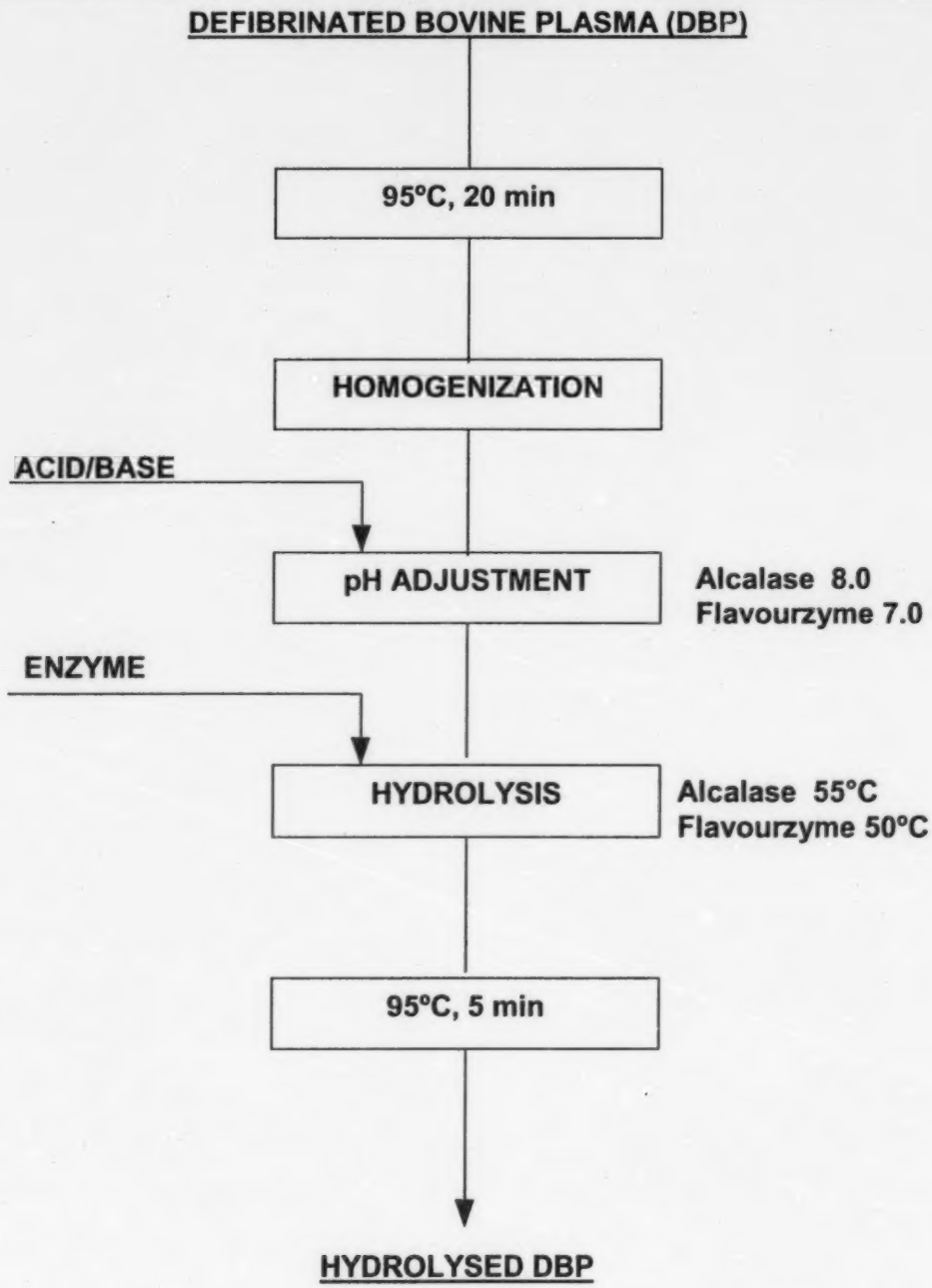

Figure 2.2 Flow chart for hydrolysis of DBP.

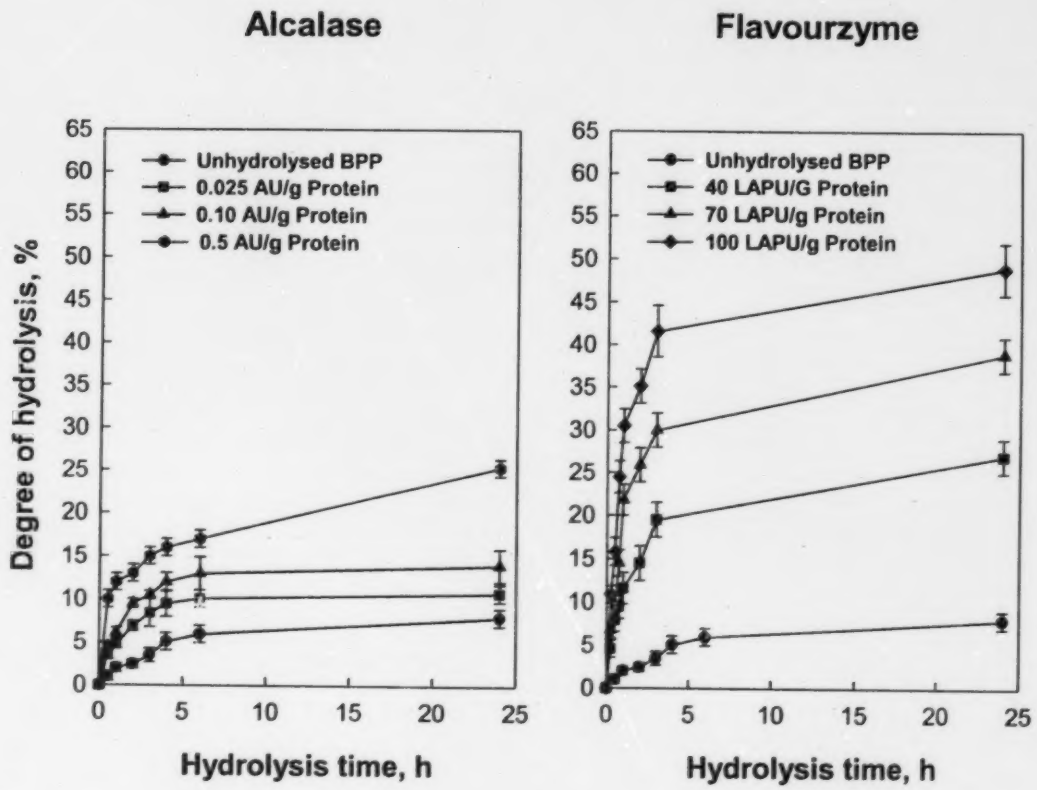

Figure 2.3 Hydrolysis of DBP proteins with Alcalase and Flavourzyme.

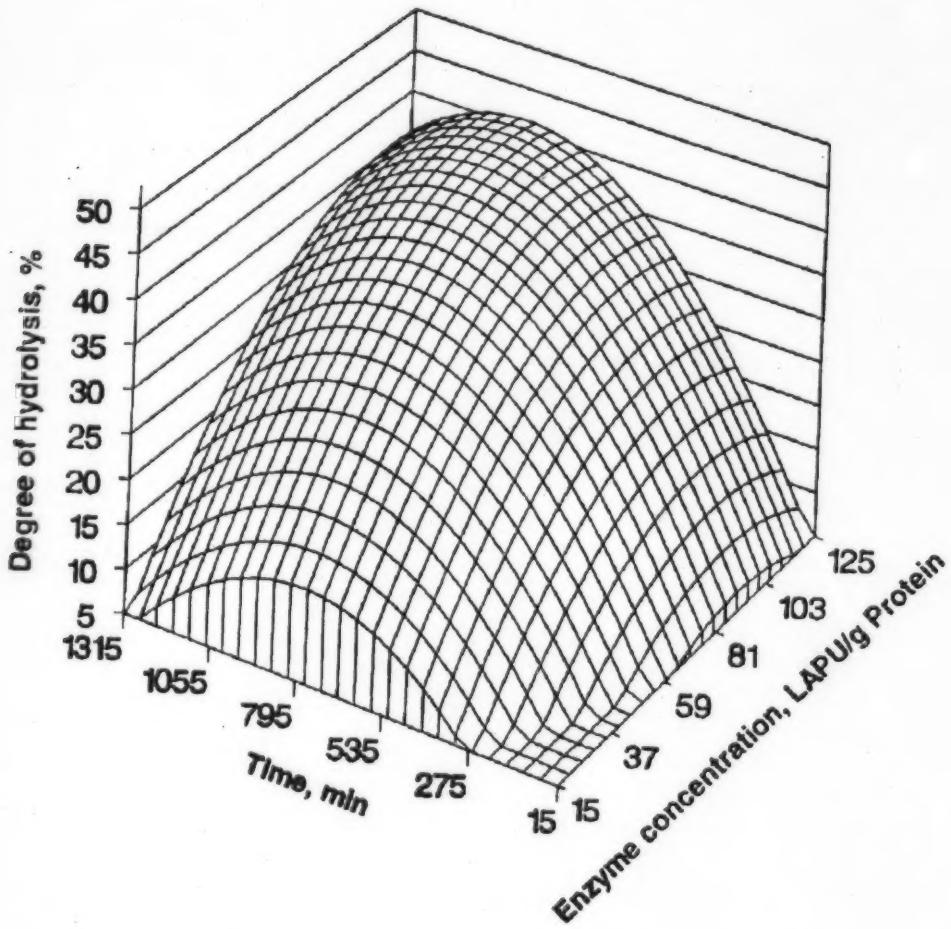

Figure 2.4 Response surface plot for degree of hydrolysis of DBP by Flavourzyme.

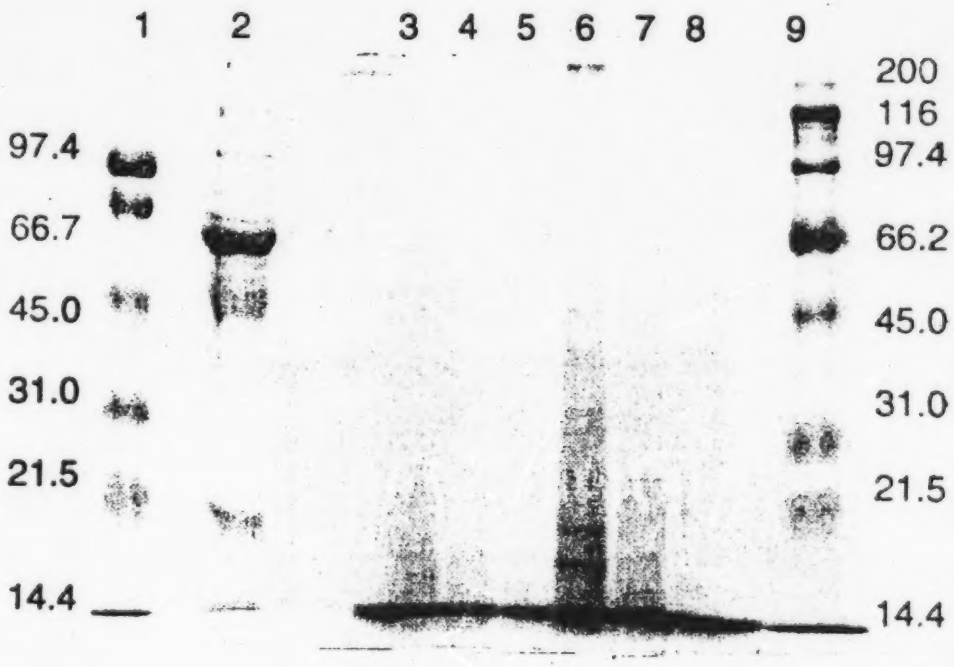

Figure 3.1 Electrophoretic separation of hydrolysed and unhydrolysed DBP, Tris-glycine PAGE: lanes 1 & 9 protein markers, lane 2 unhydrolysed DBP, lane 3,4 & 5 Alcalase hydrolysed DBP (5,13 &22% DH), lanes 6,7 & 8 Flavourzyme hydrolysed DBP (5,18 & 43% DH)

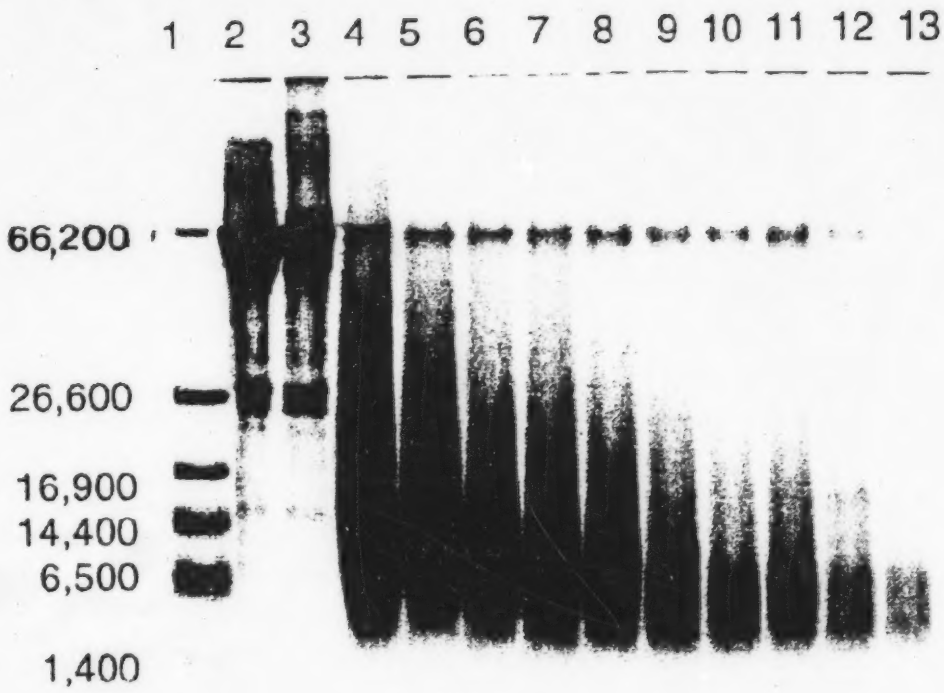

Figure 3.2 Electrophoretic separation of hydrolysed and unhydrolysed DBP Tris-tricine-PAGE: Flavourzyme-hydrolysed: lane 1 peptide markers, lanes 2 and 3 unhydrolysed DBP, lanes 4-13 hydrolysed DBP (5 to 43% DH).

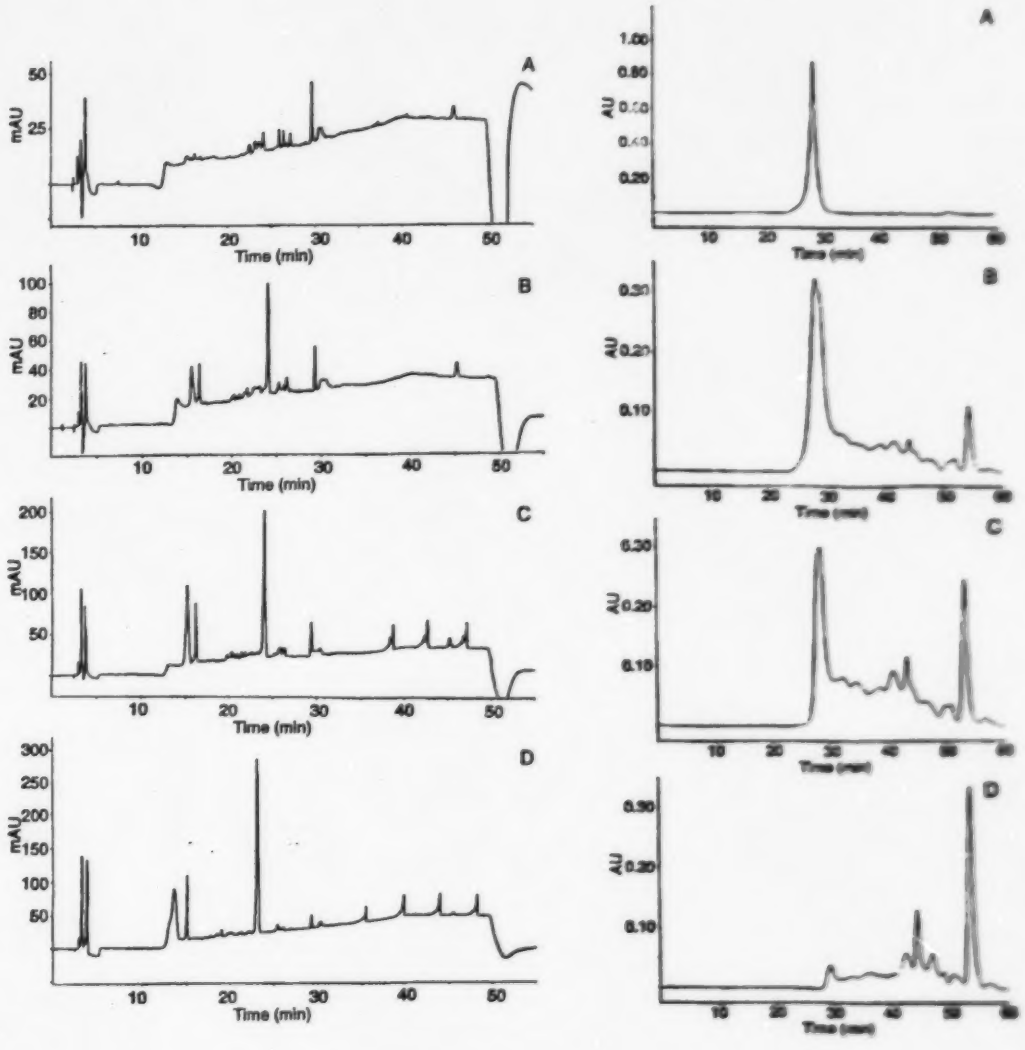

Figure 3.3 Separation of Flavourzyme hydrolysed DBP by RP-18 (1) and TSK G2500 PW (2) HPLC columns.

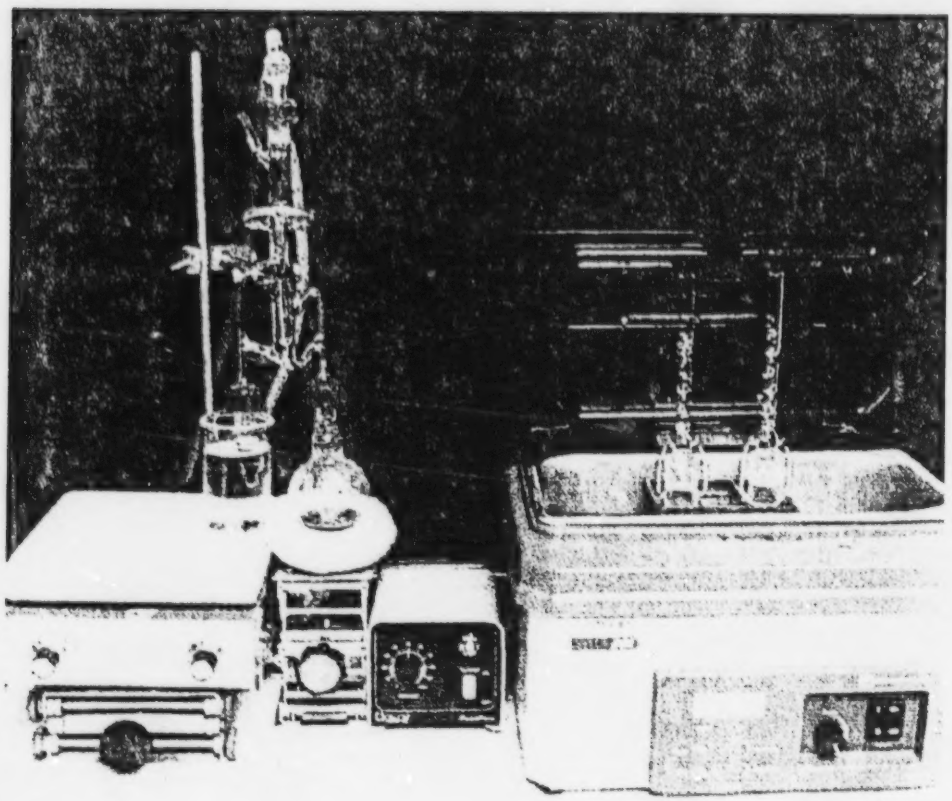

Figure 4.1 Extraction and concentration apparatus for simultaneous distillation extraction (SDE) of volatile aroma compounds.

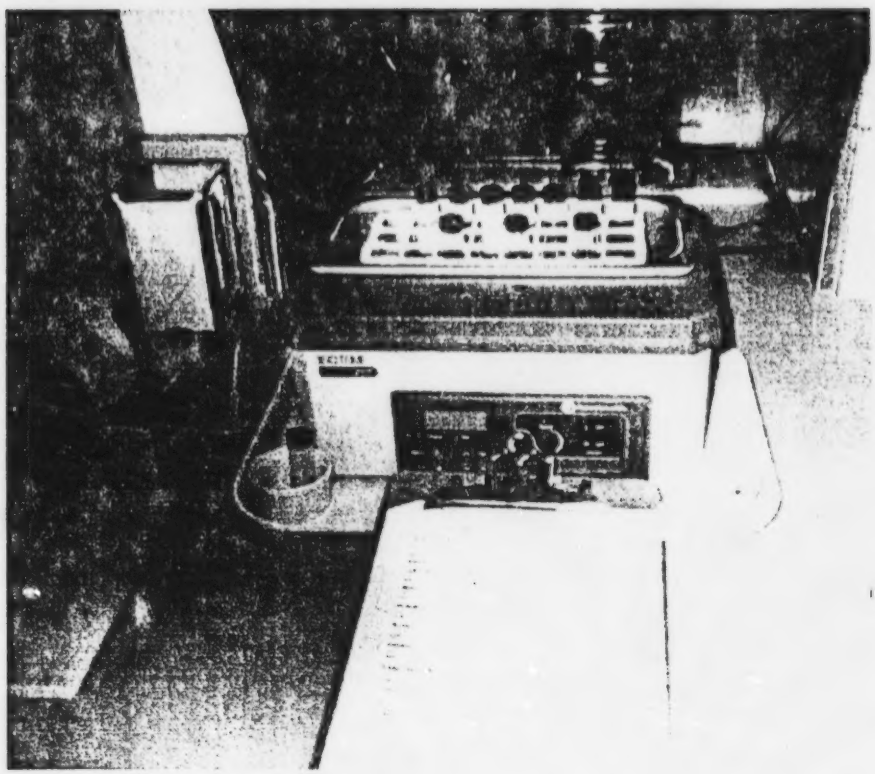

Figure 4.2 Presentation set up of sample for sensory analysis.

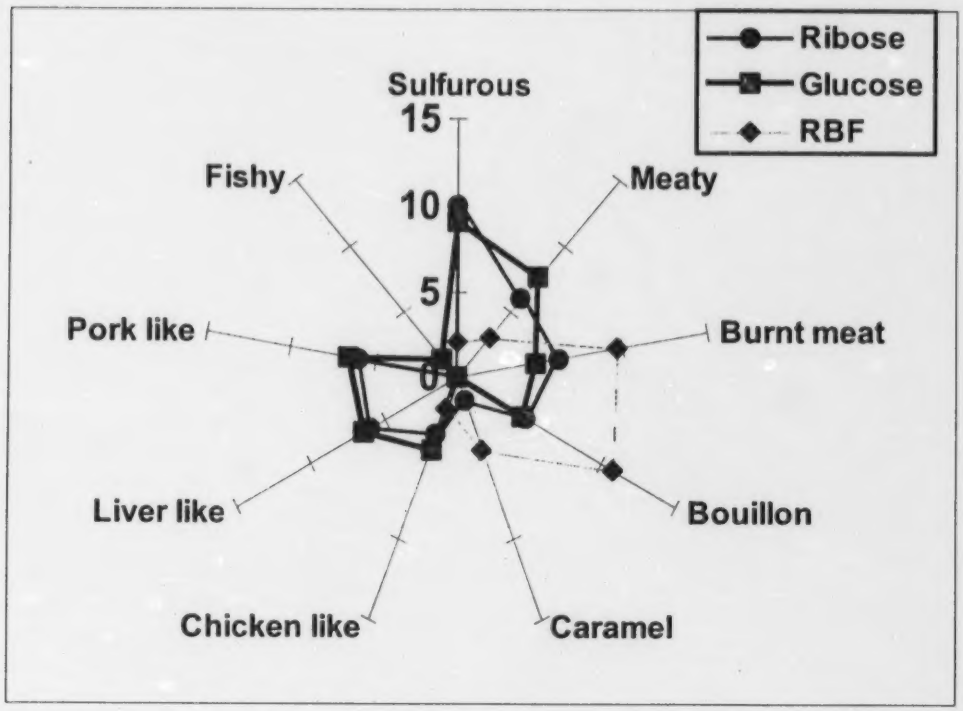

Figure 4.3 Radar chart for quantitative descriptive analysis of DBP-hydrolysate containing reaction mixtures.

APPENDIX

Quantitative Descriptive Analysis Sheet for
Odour characteristics of simulated cooked-meat flavorants

Indicate intensity of each odour descriptor of the sample by marking a "×" sign in the box chosen.
There are 15 boxes for each line.
Use the box "none" if you do not perceive the particular odour at all.

Name: _____ Date/Time: _____ Sample:_____

Sulphurous

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Meaty

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Burnt meat

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Bouillon

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Caramel

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Cooked chicken like

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Cooked liver like

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Cooked pork like

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Fishy

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong none

Evaluation Sheet for Reference Standards

Indicate intensity of odour descriptor of each reference standard provided by marking a "×" sign in the box chosen.
There are 15 boxes for each line.

Name: _____ **Date/Time:** _____

Bouillon (Bovril® beef bouillon concentrate 1:1 dilution)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Caramel (Maltol 2.5%, w/v solution)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Cooked chicken like (Boiled, blended chicken breast)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Cooked liver like (Boiled, blended beef liver)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Cooked pork like (Boiled, blended pork)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Fishy (Trimethylamine 1%, w/v solution)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong

Sulphurous (Methionine 5%, w/v solution)

☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

weak strong